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Improvement of Anti-inflammatory and Antibacterial Effects of Combined Crude Drug Extracts Including Barafu on Atopic Dermatitis

Seoung Ki Jung*, Kangwon kim, Keun-Young Kim, Min Yu Song, Woo-Tae Park

Department of Orthopaedics, Bioresource Inc.37SL2 Chumdan Venture so-ro, Gwangju, Korea

***Corresponding author:** Seoung Ki Jung, Bioresource Inc.37SL2 Chumdan Venture so-ro, Gwangju, Korea, Republic of Korea, Tel: 82-10-6411-0593; E-mail: kkw689000@gmail.com

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Abstract

Introduction: Long-term use of steroid as a cure for atopic dermatitis can cause side effects. Therefore, there has been research on commercialized use of natural combined crude drug extracts as medicines recently. Against this backdrop, the study aims to investigate the clinical, anti-inflammatory, anti-bacterial effects of combined crude drug extracts including Barafu on atopic dermatitis patients.

Methods: The combined crude drug extracts used for the study include various plant bodies such as Barafu, *Phellinus linteus* (SANG HWANG Mushroom) and formulated into a cream product. Analysis of anti-inflammatory effect was conducted using cell toxicity evaluation by MTT assay, and gene expression related to anti-inflammatory was measured with primers of TNF (tumor necrosis factor)-α, COX (cyclooxygenase)-2 and IL (interleukin)-1β. Also, anti-bacterial activity was measured by conducting antibacterial activity for *Staphylococcus aureus*. Clinical effectivity evaluation was conducted through SCORing Atopic Dermatitis (SCORAD) index and patients' global assessment of clinical response, and itching sensation was assessed with 10 cm visual analogue score.

Results: As a result of analysis on the anti-inflammatory effect, Ethanol (EtOH) solvent extract had little effect on the expression of inflammation genes, while Ethyl acetate (EA) and Methylene chloride (MC) solvent extracts showed decreased expression of *TNF-α, COX-2* and *IL-1β gene*, showing anti-inflammatory effect. As for anti-bacterial effect, combined plant extract containing Barafu showed improved anti-bacterial activity compared to other natural single plant extracts. Also, the clinical symptom evaluation showed significant decrease in objective SCORAD index before and after treatment.

Conclusion: Based on the results, it was revealed that combined plant extracts and the application products including Barafu had anti-inflammatory and anti-bacterial effects and improvement of itchiness for atopic dermatitis patients. Therefore, follow-up studies on moisturizing effect and clinical stability for patients are expected to be contributable for atopic dermatitis medicines.

Keywords: Atopic dermatitis; Anti-inflammatory effect; Anti-bacterial activity; Allergic rhinitis; Chronic urticaria

Introduction

Mainly occurring in infants and children, atopic dermatitis is chronic recurrent inflammatory dermatologic disease, accompanying severe itching sensation and skin eczema. It is known as one of the representative allergic diseases along with asthma, allergic rhinitis and chronic urticaria [1]. Atopic dermatitis starts from infant eczema through local portions including inner elbow side and posterior knee side in children to the whole body in adulthood, showing aggravation of symptoms and chronic progress. Recently in Korea, it has occurred in 34% of kindergarteners and 25% of elementary school students, and the rates continue to rise [2].

The biggest characteristics of atopic dermatitis are severe itching sensation and extremely sensitive reactivity to external stimulus or allergic provocating agents. Scratching of skin due to itching sensation can result in eczematous changes of skin; acute eczema in head, face, abdomen and the upper and lower extremity that forms red, moist and greasy scabs; eczema in forehead, periophthamic, periauricular and extremity flexure portion that thickens and dry the skin; xeroderma; papillary eczema of hand and foot, lichenoid keratosis, etc. [3].

The purpose of treatment of atopic dermatitis is to relieve symptoms, prevent the aggravation of lesions by starting treatment in the early stage and avoiding recurrence as much as possible; ultimately, it is to control the progress of disease and help patients live a normal social life without any inconvenience. There are several treatment methods for atopic dermatitis including basic measures to protect skin, conventional treatment for moisturizing and reducing itching sensation, dermatitis treatment and identifying and removing provocating agents [4,5].

However, there is no fundamental resolution for curing atopic dermatitis 100% for now. In particular, when it comes to the most widely used method, steroid treatment, they should take more precautions due to its safety issues; continuous use of steroid components may cause serious problems. Potential side effects of steroids include: adverse skin reactions such as skin atrophy, telangiectasia, hypopigmentation, steroid acne, increased hair growth and rosacea-like eruption as well as side effects throughout the body including suppression of HPA (hypothalamic-pitutary-adrenal) axis,

growth retardation, increased risk of glaucoma and cataract and Cushing's syndrome [6].

Accordingly, there have recently been active research projects on medicines for atopic dermatitis and skin diseases using natural products from plant sources with minimized skin irritation, instead of steroid components and harmful substances to skin. Major treatment principles of natural products from plant sources include reinforcement of skin moisture, regulation of cell immune status, anti-bacterial and anti-inflammatory effects [7].

Barafu is *Mesembryanthemum crystallinum* called 'crystal' in Swahili language. In order to survive harsh environmental conditions, the African desert plant forms bladder cells to save abundant water and various nutrients including beta-carotene and vitamins; which is known to have excellent effect for antioxidant of skin cells and moisturizing [8].

For atopic dermatitis patients, it is important to properly adjust cell immune responses to resist allergen and prevent immune hypersensitivity at the same time. According to research, acidic Proteo-heteroglycan from *Phellinus baumii* extract promote proliferation of spleen cells and activate lymphocytes to control cellular immunity [9,10], and phytoncide, tetranortriterpenoid, etc. from Hinoki cypress extracts prevent T cells from being excessively activated and control cell immune hypersensitivity reaction [11].

Infection with hazard bacterial including *Staphylococcus aureus* increases IgE antibodies, which stimulates mast cells, and generates inflammatory cytokines such as IL-4 and IL-5, resulting in aggravated atopic symptoms like inflammation and itching sensation. Antibiotic materials such as hexadecanoic acid contained in *Plantago asiatica* extract inhibit proliferation of harmful bacteria such as *Staphylococcus aureus* [12], *Portulaca oleracea L.* extract restrains generation of inflammation-causing cytokine and alleviates inflammation and itching sensation [13], and Fig tree extract has antibacterial activity against methicillin-resistant *Staphylococcus aureus* [14].

Besides, Aloe vera leaf extract, *Kochia scoparia* extract, White willow peel extract, Bamboo extract, *Xanthii fructus* extract, Germinated brown rice extract and others provide various nutrients improving skin conditions; *Ulmus campestris* extract, *Sciadopitys verticillata* root extract, etc. improve skin resistance; *Pulsatilla koreana* extract, *Usnea barbata* extract, *Zanthoxylum piperitum* fruit extract and others can be used for natural preservatives without skin irritation or toxicity [15-19].

However, there have been few research efforts for commercialization of such natural combined crude drug extracts; in particular, assessment on products containing Barafu have rarely been conducted. Therefore, the study aims to confirm the anti-inflammatory and anti-bacterial effect and clinical effects of combined crude drug extracts including Barafu for atopic dermatitis patients.

Materials and Methods

Combined crude drug extracts and cream products

The combined crude drug extracts used for the study were created by freeze-drying various plant bodies (Table 1) including Barafu and *Phellinus linteus* (SANG HWANG Mushroom), mixing them together and extracting with each solvent of Ethanol (EtOH), Ethyl acetate (EA) and Methylene chloride. Rotary evaporator was used to remove extracted solvent from each solvent extract, and which was diluted with distilled water to be used for the study. The cream products were provided by Bioresource® company; they were combined crude drug extracts formulated by adding ceramide, shea butter, etc.

S.no	Plant bodies
1	*Mesembryanthemum crystallinum*
2	*Phellinus linteus*
3	*Chamaecyparis obtusa* Leaf
4	*Portulaca oleraceat*
5	*Plantago asiatica*
6	*Ficus carica* (FIG) Fruit/Leaf
7	*Aloe barbadensis* Leaf
8	*Kochia scoparia* Fruit
9	*Bambusa vulgaris*
10	*Opuntia tuna* Fruit/Malt
11	*Ulmus campestris* (Elm)
12	*Salix alba* (Willow)
13	*Sciadopitys verticillata* Root
14	*Lespedeza bicolor* Bark
15	*Zanthoxylum piperitum* Fruit
16	*Pulsatilla koreana*
17	*Usnea barbata* (Lichen)
18	*Xanthium strumarium* Fruit
19	*Oryza sativa* (Rice)

Table 1: The list of herbs used.

Analysis of anti-inflammatory effect:

Cell toxicity evaluation by MTT assay: Cell toxicity of combined crude drug extracts were assessed using MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [20]. The cells used for the experiment (HaCaT, Human keratinocyte) were purchased from Korean Cell Line Bank and cultured in Roswell Park Memorial Institute medium (RPMI, Invitrogen) to which 10% fetal bovine serum (FBS, Invitrogen) and 50 μg/mL straptomycin (Sigma, USA) were added under the condition of 37^0C, 5% CO_2. Later, the cells were seeded in 96 well plates (1×10^5 cells/well) and cultured for 24 hours under the condition of 37^0C, 5% CO_2 to attach the cells. After culturing, the media were removed and the samples were processed in diluted solution according to proper concentrations and cultured for 24 hours; after adding 100 μl of 3 mg/mL MTT solution to each well, they were cultured 4 hours more. And then supernatant liquid was removed, 150 μl of Dimethyl Sulfoxide (DMSO) was added to the samples. After melting generated formazan by shaking for 30 minutes, absorbance was measured at 540 nm using multi-microplate reader (moledular device, spectra max 190) to get cell survival rates.

Anti-inflammatory effect evaluation: Anti-inflammation related gene expression were measured with Reverse Transcriptase Polymerase

Chain Reaction (RT-PCR) method using primers of inflammation-related genes including TNF (tumor necrosis factor)-α COX (cyclooxygenase)-2 and IL (interleukin)-1β (Table 2).

Gene	Primer sequence	
β-Actin	Forward	5' - CTG GGA CGA CAT GGA GAA AA - 3'
	Reverse	5' - AAG GAA GGC TGG AAG AGT GC - 3'
TNF-α	Forward	5' - ATG AGC ACT GAA AGC ATF ATC - 3'
	Reverse	5' - TCA CAG GGC AAT GAT CCC AAA GTA GAC CTG CCC - 3'
COX-2	Forward	5' - TTC AAA TGA GAT TGT GGA AAA ATT GCT - 3'
	Reverse	5' - AGA TCA TCT CTG CCT GAG TAT CTT - 3'
IL-1β	Forward	5' - CTG TCT GAA TCA GAA ATC CTT CTA TC - 3'
	Reverse	5' - CAT GTC AAA TTT CAC TGC TTC ATC C - 3'

Table 2: Primers used to qRT-PCR analysis.

After seeding cultured HaCaT cells in 6 well plates (5×10^5 cells/well), the cells were attached by culturing for 24 hours under the condition of 37^0C, 5% CO_2. Later, the media were removed, the samples of combined crude drug extracts were processed by concentration and cultured for 24 hours, and RNA analysis was conducted. Intercellular total RNAs for RNA analysis were extracted using Trizol reagent (Invitrogen®, USA); the purity and integrity of RNAs were investigated by measuring the A260 nm/A280 nm ratio, and the RNA yield fabrication was measured with absorbance at 260 nm.

For cDNA synthesis, Oligo dT 15 (500 ng/μl) primer, dNTP (10 mM), RTase inhibitor (40 units/μL) and Powerscript RTase were added to 1 μg of total RNAs, and which was primer-annealed at 25 for 10 minutes. And then, cDNA was synthesized at 42^0C for 60 minutes and RTase denaturation was proceeded at 95^0C for 5 minutes.

In order to amplify β-Actin, TNF-α, COX-2 and IL-1β from the cDNA, Light cycler 96 (Roche®, Germany) was used to proceed real-time PCR as follows: after putting in 2 μL of cDNA template, Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen®, USA), 5 pmol of sense and anti-sense primer (Table 2), ROX reference dye and bovine serum albumin, distilled water was added to make the total volume 20 μL. For PCR thermo-circulation, it was pre-denatured at 95 for 2 minutes. During 30 cycles, denaturation at 94^0C for 5 seconds, annealing at 55^0C for 20 seconds and extension at 72^0C for 10 seconds were conducted and the final extension at 72^0C for 5 minutes. The result was estimated as expression of β-Actin to relative expression.

Anti-bacterial activity against *Staphylococcus aureus*

Staphylococcus aureus (KCTC 3881) strain for anti-bacterial experiment was given by Korean Collection for Type Cultures. Through 3 times of subculture at 37^0C for 24 hours in nutrient media, it was activated and used for the vaccinated strain. Anti-bacterial activity was measured with the method of paper disc [21]. After smearing pre-cultured vaccinated solution in agar plates of nutrient media, a paper disc where 1 g samples of *Hinoki cypress* extract, Fig tree extracts and combined crude drug extracts were dropped was placed and cultured at 37^0C for 16 hours. And then, the size of clear zone (mm) around the paper disc was measured to evaluate the anti-bacterial activity.

Clinical effectivity evaluation:

1) Subject of study: The study recruited 50 patients aged 2-13 (28 males and 22 females) who met the diagnosis standard of atopic dermatitis by Hanifin and Rajka [22] and had not received treatment for the last 4 weeks from a multi dermatologic clinic center between the 15th Feb and the 9th May 2016 (Table 3). After explanation about the purpose and schedule of the clinical study and possible risks, those who agreed in written form to participate in were included in the study. However, patients who had sever skin diseases or body diseases aside from atopic dermatitis and those who were classified in the severe level according to Raika and Langeland and [23] were excluded.

2) Clinical evaluation: The registered patients visited the department of dermatology before testing, on the treatment day and 4-week, 8-week and 12-week after treatment (on the last day of treatment). On the first day of treatment, they got physical check-ups and were questioned about previous medical history and itchiness level and examined for all skin lesions. They were made to apply a proper amount of the cream product twice a day after washing face or showering on dry lesion during the testing period.

Objective SCORAD index: Clinical improvement degrees of the patients were measured by two dermatologists in a 4-week interval before and after treatment by the end of treatment using Objective SCORAD index [24]. Objective SCORAD index is used for cases that subjective symptoms such as itchiness and sleeping disturbance level are difficult to objectify for child patients as in the study. Aside from subjective symptoms, objective factors such as range of lesion (extent %) and intensity of erythema, exudate, abrasion, fissure, xeroderma and lichenoid keratosis were evaluated between 0 and 3 points, and each score was summed up.

Evaluation of the patients' clinical response, itching sensation and adverse effect: On the visits of 4, 8 and 12-week except for the first day of treatment, the patients or the same guardian of them assessed patients global assessment of clinical response (S1; Very much improved, S2; Much improved, S3; Improved, S4; Stationary, S5; Worse, S6; Much worse, S7; Very much worse) and 10 cm visual analogue score for itching sensation (0 cm: not itchy at all, 10 cm: the worst itchiness you can imagine) and reported any side effects occurred after treatment.

Result

Cell toxicity evaluation

Before the anti-inflammatory effectivity experiment on the combined crude drug extracts, a cellular toxicity evaluation was carried out through MTT assay. MTT assay is a technique to measure cell proliferation or living cells accurately using the capacity of mitochondria that reduces MTT tetrazolium, yellow water-soluble substrate to celadon green and anti-water soluble MTT formazan (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide) through dehydrogenated enzyme reaction. Absorbance of MTT formazan peaks at wave length of 540 nm, where the measured absorbance reflects the concentration of living and metabolically active cells [17].

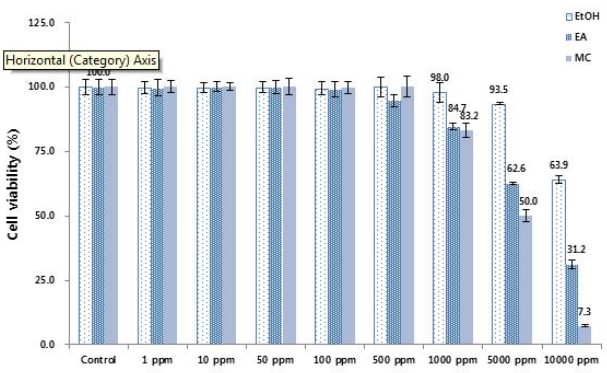

Figure 1: Cell viability of human keratinocyte treated various concentration of herb complex extracts using EtOH, EA and MC. Error bars show standard deviation values.

Figure 1 shows the combined crude drug extracts' cell toxicity against human keratinocyte. As for EtOH extract, cell toxicity was revealed at concentration over 10000 ppm, and MC and EA extracts showed at over 5000 ppm of concentrations. Cell viability of extract at 1000 ppm was EtOH 98.0%, EA 84.7% and MC 83.2%, based on which, the anti-inflammatory effect evaluation of combined crude drug extracts for human keratinocyte was conducted with concentration below 1000 ppm where there was no cell toxicity.

Anti-inflammatory effect evaluation

Anti-inflammatory effect evaluation was conducted by checking the relative expression of *β-Actin* of genes that are related to cause of inflammation such as *TNF-α, COX-2* and *IL-1β* using qRT-PCR, with concentration below 1000 ppm where no toxicity was observed in the cell toxicity evaluation.

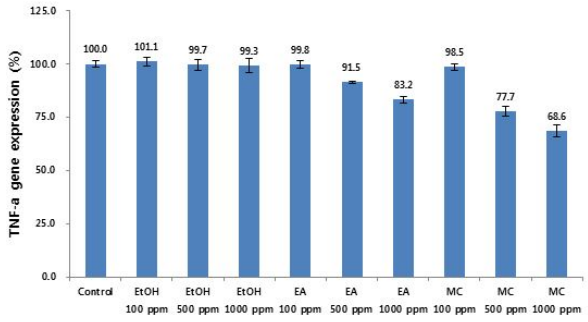

Figure 2: Expression of *TNF-α* gene in human keratinocyte treated various concentration of herb complex extracts using EtOH, EA and MC. Transcription levels were analyzed relative to that of β-Actin. Error bars show standard deviation values.

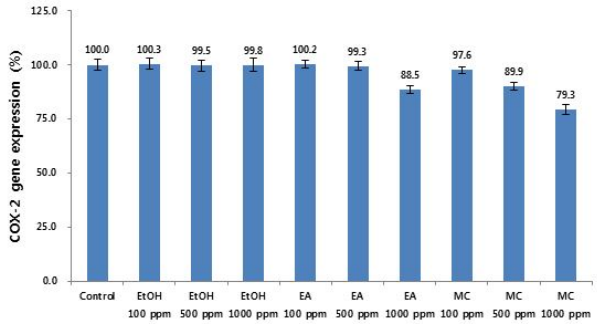

Figure 3: Expression of *COX-2* gene in human keratinocyte treated various concentration of herb complex extracts using EtOH, EA and MC. Transcription levels were analyzed relative to that of β-Actin. Error bars show standard deviation values.

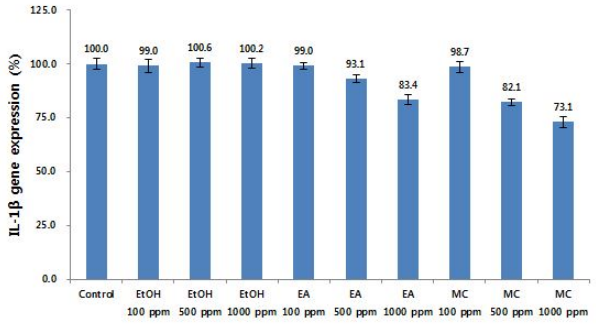

Figure 4: Expression of *IL-1β* gene in human keratinocyte treated various concentration of herb complex extracts using EtOH, EA and MC. Transcription levels were analyzed relative to that of β-Actin. Error bars show standard deviation values.

Figures 2-4 shows gene expression results of each TNF-α, COX-2 and IL-1β. EtOH extract had little impact on expression of genes related to inflammation, while cells processed with EA and MC extracts showed that the expression of *TNF-α*, *COX-2* and *IL-1β* genes concentration-dependently reduced.

Among inflammation-related cytokines, *TNF-α*, *IL-1β*, etc. are connected with various immunological reactions as pro-inflammatory cytokines [25,26]. Especially, TNF-α has an important role in inflammation reactions. It is produced from macrophage, mast cells, etc., plays an important role in intrinsic immune as a major medium of LPS (lipopolysaccharide) reaction, and is related to chronic inflammation [25]. Meanwhile, IL-1β activates T-cell, maturation of B-cell and activity of NK cell, and is reported to always increase in inflammatory lesion [26]. COX-2 plays a part in the synthesis of prostaglandins in acute inflammatory reaction, and its expression is induced by LPS (Lipopolysaccharide) and cytokine [27,28].

Therefore, reduced expressions of *TNF-α*, *COX-2* and *IL-1β* genes are considered to be effective for curing inflammatory diseases by

Barafu and other combined crude drug extracts inhibiting cytokines including *TNF-α*, *COX-2* and *IL-1β* that are related to inflammation.

Anti-bacterial effect

Anti-bacterial activity of *Hinoki cypress* extract [11], Fig tree extracts [14] and combined crude drug extracts that are known to have anti-bacterial activity for *Staphylococcus aureus* was evaluated by measuring the size of clear zone (mm) around the paper disc where extracts were dropped; the evaluation results of anti-bacterial activity for *Staphylococcus aureus* are shown in Table 4 and Figure 5.

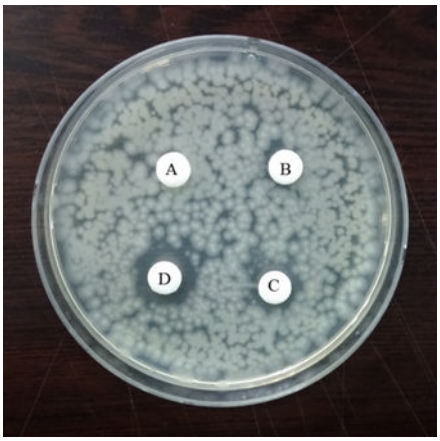

Figure 5: Antimicrobial activities for various herb extract from *Chamaecyparis obtusa* leaf, *Ficus carica* (FIG) fruit/leaf and herb complex against *Staphylococcus aureus*; (A) Control (D.W), (B) *Chamaecyparis obtusa* leaf extract, (C) *Ficus carica* (FIG) fruit/leaf extract, (D) Herb complex extract.

As a result, the anti-bacterial activity for *Staphylococcus aureus* had differences by each extract as shown in Figure 5; 1 g of *Hinoki cypress* extract and Fig tree extract samples showed anti-bacterial activity in the range of 10 mm and 12 mm. On the other hand, combined plant extract had anti-bacterial activity in the range of 15 mm, showing improved anti-bacterial activity compared to single plant extract (Table 4).

The medical community have reported that more than 90% of atopic patients are detected with yellow *Staphylococcus aureus* in skin, and the bacteria creates toxicity aggravating skin diseases including atopic dermatitis [29]. Therefore, as identified in the study results, Barafu and other combined plants extracts can be used to make atopic skin disease medicines with excellent effects as well as in other industries.

Clinical symptom evaluation

There are several ways to evaluate severity of atopic dermatitis, but the study employed the most well-known method, SCORing Atopic Dermatitis (SCORAD) index reported by the European Task Force on Atopic Dermatitis [30] for the evaluation.

A total of 50 patients including 28 boys (56%) and 22 girls (44%) aged 2-13 were recruited for the study. The subjects consisted of 11 children aged under 4 (22%), 14 aged 5-7 (28%), 14 aged 8-10 (28%) and 11 aged 11-13 (22%), and their prevalence duration was 1-11 years. Results of 45 out of the 50 patients were used for the evaluation, excluding 5 for whom tracking observation had not been conducted on a regular basis.

Before treatment, the average objective SCORAD index of the 45 patients was 38.5, which reduced to 22.34 after 4-week treatment, 17.18 after 8-week, and 15.42 after 12-week; they showed significant decreases in objective SCORAD index compared to before treatment (Figure 6). Also, most patients showed distinct mitigation of symptoms that could be checked with naked eyes (Figure 7).

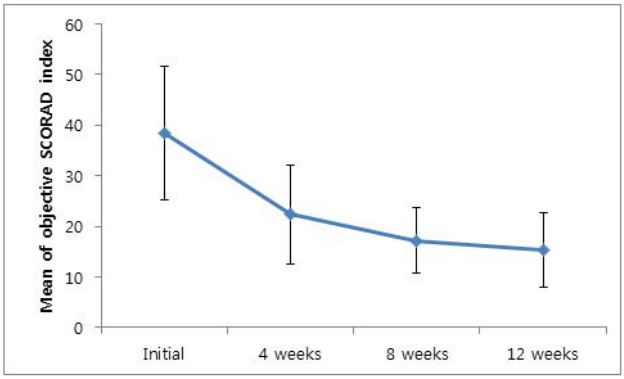

Figure 6: The mean of objective SCORAD index of 45 patients.

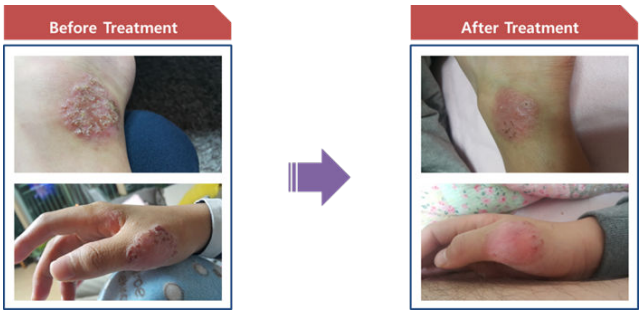

Figure 7: Significant clinical dermatologic symptom change after cream applyment.

The patients' clinical symptoms response evaluation scores improved during the whole period of treatment, and 42 of them (93.3%) evaluated the treatment higher than 'Improved' at the end of the treatment. The remaining 3 patients (6.7%) evaluated 'Stationary' (Figure 8). The evaluation scores for itchiness continuously decreased from 7.5 at the beginning of the treatment to 4.1 at the end for the 12 weeks (Figure 9).

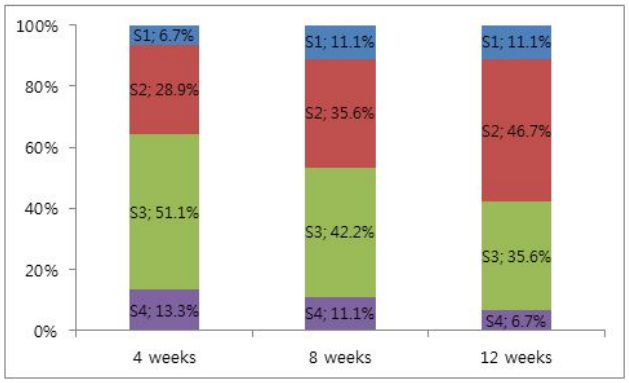

Figure 8: Change from baseline to the end of treatment for percentage of patient's global assessment of clinical response (S1; Very much improved, S2; Much improved, S3; Improved, S4; Stationary)

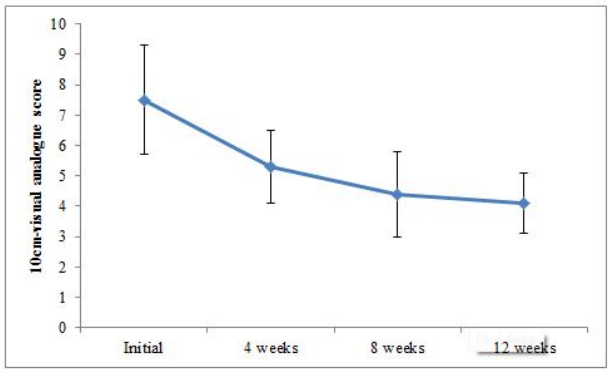

Figure 9: Changes of pruritus scores by patients.

The study results were similar to that of clinical experiments using *Phellinus linteus* [24,30], Bamboo tree [16], Germinated brown rice [24], Green tea [7] extract and muti-variable plants extracts. The study confirmed that Barafu and other combined plant extracts have much better effects than single plant extracts.

Conclusion

Based on the results, it was confirmed that combined plant extracts including Barafu and the application products reduced SCORAD index and itching sensation, had anti-inflammatory and anti-bacterial effects for atopic dermatitis patients. Also, it was revealed that they were excellent in moisturizing the lesion during the clinical experiment. However, objectification research has not been conducted yet, objectified follow-up studies on the moisturizing effect and comparison analysis between placebos and existing products to prove the effects of Barafu and other combined plant extracts are expected to contribute to developing medicines for atopic skin diseases.

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